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ADMP2 is essential for primitive blood and heart development in *Xenopus*

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Abstract

We describe here the cloning of a new member of the TGF-β family with similarity to the anti-dorsalizing morphogenetic proteins (ADMPs). This new gene, ADMP2, is expressed in a broad band of mesendoderm cells that appear to include the progenitors of the endoderm and the ventral mesoderm. Antisense morpholino oligonucleotide knockdown of ADMP2 results in near-complete disruption of primitive blood and heart development, while the development of other mesoderm derivatives, including pronephros, muscle and lateral plate is not disrupted. Moreover, the development of the primitive blood in ADMP2 knockdown embryos cannot be rescued by BMP. These results suggests that ADMP2 plays an early role in specifying presumptive ventral mesoderm in the leading edge mesoderm, and that ADMP2 activity may be necessary to respond to BMP signaling in the context of ventral mesoderm induction.

Keywords: Xenopus; Mesoderm; Blood

Introduction

Xenopus laevis has long served as a model for vertebrate mesoderm patterning. Multiple determinative and inductive mechanisms are responsible for patterning the mesoderm along the dorsal/ventral, anterior/posterior and left/right axes. Dorsoventral patterning is evident in the larval trunk, with notochord situated at the dorsal midline, somites located ventral and lateral to the notochord, followed by pronephros and lateral plate, and finally, the blood islands occupying the ventral-most region. Models have attempted to explain dorsoventral mesoderm patterning in *Xenopus* through the action of the Spemann organizer. Most simply, it was proposed that a gradient of induction arising from the Spemann organizer would specify mesodermal identities due to the relative positions of the presumptive mesodermal cells in the gastrula marginal zone (Kessler and Melton, 1994; Harland and Gerhart, 1997; Heasman, 1997; Dale and Jones, 1999; Dale and Wardle, 1999; De Robertis et al., 2000; De Robertis and Kuroda, 2004). Accordingly, cells closest to the Spemann organizer would be specified to dorsal fates such as somite, while those cells furthest away from the Spemann organizer would be specified as ventral, such as blood island.

Despite a wealth of data consistent with the Spemann organizer model of dorsoventral pattern, recent studies have raised questions regarding this model (Lane et al., 2004; Kumano and Smith, 2002b). First, the mesoderm precursors are not distributed in a simple dorsal to ventral array with respect to the Spemann organizer in the gastrula embryo. The posterior somites, for example, are derived from mesoderm that is at the opposite side of the marginal zone from the Spemann organizer (the contra-organizer marginal zone) (Dale and Slack, 1987; Moody, 1987; Lane and Smith, 1999), while anterior blood islands are derived from mesoderm that is immediately adjacent to the Spemann organizer (Tracey et al., 1998; Lane and Smith, 1999; Mills et al., 1999; Ciau-Uitz et al., 2000). Second, a great deal of patterning of the marginal zone is observed in the absence of the Spemann organizer, including the restriction of the blood islands to the presumptive ventral mesoderm (Kumano and Smith, 2000), and the restriction of myoD expression to a domain in the early gastrula embryo that appears to correspond to the presumptive somites (Kumano and Smith, 2002b).

These difficulties with the Spemann organizer model of mesoderm patterning have led to a hunt for alternative models (Kumano and Smith, 2002b). One key early activity for

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specifying mesoderm identity in the dorsoventral axis appears to be FGF. Experimental reduction of FGF activity with a dominant negative FGF-receptor results in expansion of the blood islands with a corresponding loss of somites (Kumano and Smith, 2000). We have shown that manipulation of FGF signaling alters the response of *Xenopus* animal cap ectoderm explants (animal caps) to the mesoderm inducer *nodal* in a way that appears to mirror induction in the gastrula marginal zone (Kumano et al., 2001). Whereas exogenous *Xenopus* nodal related-2 (Xnr2) alone induces dorsal mesoderm (skeletal muscle) in animal caps, inhibition of endogenous FGF signaling in the animal cap with a dominant negative FGF receptor (XFD) results in the induction of ventral mesoderm (primitive blood) in response to exogenous Xnr2. It has been described previously that animal caps have elevated FGF signaling due to the injury caused by dissection (LaBonne and Whitman, 1997; Christen and Slack, 1999). The animal cap thus represents a highly tractable model for investigating the mechanisms that pattern mesoderm in the gastrula embryo. We report here results from a subtraction cloning project aimed at identifying genes that are induced in animal caps by Xnr2 in the absence of FGF signaling. We describe the identification of a new member of the transforming growth factor-β (TGF-β) family, *Xenopus* anti-dorsalizing morphogenetic protein 2 (ADMP2). ADMP2 is expressed endogenously at gastrulation in a broad band of cells that appears to include the endoderm and the leading edge mesoderm. Antisense morpholino oligonucleotide knockdown of ADMP2 resulted in disruption of the development of the blood islands and heart, both ventral mesoderm derivatives, but had no apparent effect on the development of other mesoderm derivatives such as somite and lateral plate.

Materials and methods

Xenopus embryos

Ovulation was induced by injecting female *X. laevis* with human chorionic gonadotropin (Sigma) and eggs were fertilized as described previously (Condie and Harland, 1987). Eggs were dejellied 20 min after fertilization in 2% cysteine–HCL (pH 8.0), and were cultured in 1/3 MMR until ready for injection and RNA extraction. Embryos were staged according to Nieuwkoop and Faber (1994).

Isolation of Xenopus ADMP2 by subtraction and 5′ RACE

ADMP2 was identified by nucleotide sequencing of randomly picked clones from a previously described subtracted library (Kumano and Smith, 2002a). A full-length cDNA was isolated by 5′ RACE using the SMART RACE cDNA Amplification Kit (CLONTECH) and the oligonucleotide (5′-ATGGCACCCA-CAGCTGACAGCAATCA-3′) as a gene-specific primer.

Plasmid constructs, RNA synthesis and microinjection

In vitro transcription constructs for microinjection in the vector pSP64T3 (Thomsen and Melton, 1993) were prepared as follows. For ADMP2, a fragment containing the 5′ UTR, the entire coding region and a partial 3′ UTR of ADMP2 was PCR-amplified with the upstream oligonucleotide (5′-GTACGGTACCA-TAGATGACACTTGACCAACCACTGGGAAAGTT-3′), which added a *Kpn*I site at the 5′ end, and the downstream oligonucleotide (5′-CAACAAGGATCCT-GACCAGGCAGGTATATAGA-3′), which added a *Bam*HI site at the 5′ end. The fragment was then digested with *Kpn*I/*Bam*HI and subcloned into the *Kpn*I and *Bgl*II sites of pSP64T3. An ADMP construct was made using the same strategy with the exception that the upstream *Kpn*I-containing oligonucleotide (5′-CACAAGGTACCTCGAGAGCTGCAGCTTGATGAGATGGA-3′) and the downstream *Bgl*II-containing oligonucleotide (5′-TCTATAGATCTCCC-TAGTTTGCACCTTAGACAGTACCACCA-3′) were used, and that the amplified fragment was digested with *Kpn*I/*Bgl*II.

A GFP-tagged version of ADMP2 (A-GFP) was made to test the activity of the morpholino oligonucleotide. For this construct, a fragment containing the 5′ UTR and the first 6 nucleotides of the coding region of ADMP2 was PCR-amplified using the *Kpn*I-containing oligonucleotide described above and 5′-AATAGAGCTCGCACATCCTTGCTTCTTGGGTGAAAGTTGCCT-3′, which added a *Sac*I site at the 5′ end. The fragment was then digested with *Kpn*I/*Sac*I. A GFP cDNA in pSP64T3 was prepared by excising the Xmenf cDNA from previously described GFP-tagged version of Xmenf (Kumano and Smith, 2002a) by *Kpn*I/*Sac*I digestion. The *Kpn*I/*Sac*I digested ADMP2 fragment was then ligated into the *Kpn*I and *Sac*I sites of the GFP-containing vector. The resulting construct has additional Glu-Leu residues inserted between the ADMP2 and GFP coding regions.

An ADMP2 construct in which the entire 5′ UTR is deleted (ADMP2 Δ-5′ UTR) was made for morpholino oligonucleotide rescue experiments. A fragment that contained the entire coding region and a partial 3′ UTR of ADMP2 but not the 5′ UTR was PCR-amplified using the oligonucleotide (5′-AGAAGGTAC-CATGTGCTTGGCGTATTTCTGCTTTCTGATGCT-3′), which added a *Kpn*I site at the 5′ end and the *Bam*HI-containing oligonucleotide described above. The amplified fragment was digested with *Kpn*I/*Bam*HI and ligated into the *Kpn*I and *Bgl*II sites of pSP64T3.

Plasmids for in vitro RNA synthesis were linearized with *Eco*RI for XFD (Amaya et al., 1991), bFGF (Kimelman and Maas, 1992), tBR (Suzuki et al., 1994), ADMP and ADMP2, with *Xho*I for Xnr2 and BMP7 (Nishimatsu et al., 1992), with *Bam*HI for Xwnt8 (Christian et al., 1991), and with *Sal*I for A-GFP and ADMP2 Δ-5′UTR. Capped RNAs were transcribed with mMessage mMachine (Ambion). Embryos were injected by air pressure in 1/3× MMR, 2.5% ficoll and 25 μg/ml gentamycin. For animal cap assays, 10 nl containing 200 pg of XFD, 500 pg of Xnr2 or 200 pg of XFD plus 500 pg of Xnr2 RNAs was injected into the animal regions of the both blastomeres at the two-cell stage. 250 pg of ADMP, ADMP2 or BMP7 RNAs was injected with or without 500 pg of tBR RNA into the animal regions of the both blastomeres at the two-cell stage. Also, 100 pg of Xwnt8 RNA was injected into the same regions. Finally, 1 nl containing 200 pg of XFD RNA was injected into both the AB4 (the mother cell of A4 and B4) and CD4 (the mother cell of C4 and D4) blastomeres at the 16-cell stage.

RNA extraction and Northern blot

Total RNA was isolated from whole embryos and animal caps using Trizol Reagent. Northern analysis was performed as described previously (Kumano et al., 2001). Probes were prepared with isolated fragments from alpha T3 globin (Banville and Williams, 1985), muscle actin (Dworkin-Rastl et al., 1986) and EF1α (Krieg et al., 1989). A probe for ADMP was prepared by digesting the ADMP in vitro transcription construct with *Kpn*I/*Bgl*II. A probe for ADMP2 was prepared by digesting a pCRII plasmid containing the ADMP2 5′ RACE product with *Eco*RI.

In situ hybridization

Detection of *ADMP2*, *XmyoD* (Hopwood et al., 1989), *Xlim1* (Taira et al., 1994), *Xnr1* (Lustig et al., 1996), *endodermin* (*edd*, Sasai et al., 1996), *αT3 globin* (Banville and Williams, 1985), *SCL* (Mead et al., 1998), *Nkx2.5* (Tonissen et al., 1994) and *TnIc* (Drysdale et al., 1994) expression by in situ hybridization was performed as described previously (Moos et al., 1995). A digoxigenein-labeled probe for ADMP2 was synthesized with T7 polymerase using the plasmid that contains the ADMP2 5′ RACE product and that was linearized with *Bam*HI.

Morpholino knockdown and Western blotting

The morpholino oligonucleotide used to knockdown ADMP2 expression, A-MO (5′-CACATCCTTGCTTCTTGGGTGAAAG-3′), was purchased from

Gene-Tools LLC. The standard control (c-MO) provided by Gene-Tools was used as a negative control. c-MO or A-MO (5 or 10 ng) was injected into the marginal zone of either the anterior (lighter pigmentation) or posterior (darker pigmentation) two blastomeres at the 4-cell stage. To test the efficacy of the morpholino knock-down approach, embryos were separately injected at the animal pole with 10 ng of either c-MO or A-MO and 100 pg of A-GFP RNA. The injected embryos were cultured to the neurula stage at which point protein was extracted using the freon method to remove yolk from the lysates (Gurdon and Wickens, 1983). The proteins were resolved by 12% SDS-PAGE, transferred to nitrocellulose membrane Hybond ECL (Amersham) and blocked for 2 h at room temperature with 2% nonfat milk in 20 mM Tris–HCl pH 7.4, 150 mM NaCl and 0.1% Tween 20. Immunoblotting was performed using the chemiluminescence method (PIERCE). Anti-GFP (Santa Cruz) and anti-alpha-tubulin antibodies (Sigma) were used at a dilution of 1:500.

For the rescue experiments, 10 ng of A-MO, with or without 10 pg of ADMP2 Δ−5′ UTR RNA, was injected into the marginal zone of the posterior 2 blastomeres at the 4-cell stage, and 10 ng of A-MO with or without 5 pg of ADMP2 Δ-5′ UTR RNA was injected into the marginal zone of the anterior 2 blastomeres at the 4 cell stage. ADMP2 Δ-5′ UTR has 15 mismatches out of the 25 nucleotides for the morpholino oligo.

Finally, 250 pg of BMP7 RNA (total 1 ng) with or without 10 ng of A-MO (total 40 ng) was injected into the marginal zone of all the blastomeres at the 4-cell stage.

β-galactosidase staining

10 ng of A-MO was injected into the posterior 2 blastomeres (total 20 ng) at the 4-cell stage, and subsequently, 250 pg of βgal RNA was injected into both the A4 and B4 blastomeres (total 1 ng) of the A-MO-injected embryos at the 32-cell stage. The twice-injected embryos were cultured until stage 32 and subjected to βgal staining followed by globin in situ hybridization. βgal staining was done as described previously (Kumano et al., 1999).

Phylogenic analysis

The sequences of fugu ADMPs (*Fr*ADMP) were obtained from: http://fugu.hgmp.mrc.ac.uk. The sequences of *Xenopus tropicalis* ADMPs (*Xt*ADMP) were obtained from: http://genome.jgi-psf.org/xenopus0/xenopus0.home.html. The sequence of *Ciona intestinalis* ADMP (*Ci*ADMP; Hino et al., 2003) was obtained by searching Ghost Database: a *C. intestinalis* cDNA resource at http://ghost.zool.kyoto-u.ac.jp/indexr1.html. For those genes in GenBank, the accession numbers are as follows: *Xl*ADMP, U22155; chick ADMP (*Gg*ADMP), AF082178; zebrafish ADMP (*Dr*ADMP), AJ315468/AF418564/NM131876; rat BMP3 (*Rn*BMP3), D63860/NM017105/S77492; *Xl*BMP7, X63427/U38559; *Xl*BMP2, X63424/X63425/X55031; *Xl*BMP4, X63426/X64538. Amino acid sequences were aligned using CLUSTAL X and then the phylogenetic tree was generated by the neighbor-joining method.

Results

ADMP2 is a novel member of the TGF-β superfamily

To identify genes potentially involved in ventral mesoderm induction, a subtractive cloning approach was used to isolate genes that were induced by Xnr2 in *Xenopus* animal caps in the absence of FGF signaling (Kumano and Smith, 2002a). So far, two novel genes have been isolated by this approach. The first of the two, *Xmenf*, has been described previously (Kumano and Smith, 2002a). The second gene, and the subject of this report, is a novel member of the TGF-β family. This gene was identified by sequencing the ends of 100 randomly selected subtracted clones. The initial isolate of this gene was a partial cDNA clone (297 bp) that showed strong sequence similarity to *Xenopus*, chick and zebrafish anti-dorsalizing morphogenetic proteins (ADMPs) using a translated BLAST search (Moos et al., 1995; Joubin and Stern, 1999; Lele et al., 2001), and was named ADMP2. ADMP2 was found to be expressed more highly in RNA samples from animal caps injected with Xnr2 and dominant negative FGF receptor (XFD) RNAs versus those injected with XFD RNA alone (data not shown). The partial ADMP2 cDNA contained the stop codon and a partial 3′ UTR, but neither the 5′ UTR nor the translation start site. A full-length ADMP2 cDNA was subsequently obtained by 5′ RACE using a primer in the 3′ UTR. The 1652 bp 5′ RACE product had a 306 bp 5′ UTR, a 1200 bp open reading frame and 146 bp of 3′ UTR. The deduced amino acid sequence had several features characteristic of the TGF-β superfamily, including two consensus RXXR proteolytic processing sites (Ozkaynak et al., 1992) and seven highly conserved cysteine residues in a carboxyl terminal domain at the positions observed in other members of this family.

In nearly all TGF-βs, the C-terminal one-third of the protein constitutes the mature processed form. Accordingly, database searching and multiple pair-wise alignments of ADMP2 to other ADMP-like and BMP molecules were performed using a C-terminal domain following the first conserved cysteine residue, which approximates the mature domain. Within this region, ADMP2 showed 78.6%, 76.7% and 74.8% amino acid sequence identities with *Xenopus*, chick and zebrafish ADMPs, respectively. *Xenopus* ADMP, on the other hand, showed 94.2% and 82.5% identities with chick and zebrafish ADMPs, respectively. From a BLAST search of the unassembled sequence traces from the *X. tropicalis* genome sequencing project (http://genome.jgi-psf.org/xenopus0/xenopus0.home.html), we identified two predicted genes that appeared to be the orthologs of *X. laevis* ADMP and ADMP2. The predicted *X. tropicalis* and *X. laevis* ADMP and ADMP2 proteins had 100% and 99% sequence identity in the mature region, respectively. It is hypothesized that *X. laevis* has undergone a genome duplication following its split the lineage leading to *X. tropicalis*. The presence of ADMP and ADMP2 orthologs in both *Xenopus* species rules out that the two genes could be the product of the proposed genome duplication in *X. laevis*. Outside of the two *Xenopus* species, two separate predicted ADMP-like genes were identified in the *Fugu rubripes* (http://fugu.hgmp.mrc.ac.uk) and *Tetraodon nigroviridis* (http://www.genoscope.cns.fr/externe/tetraodon) genomes. However, a search of the zebrafish genome and ESTs yielded only a single ADMP gene. Fig. 1A shows an alignment between the mature domain of ADMP2 and other similar TGF-β family members.

Phylogenetic analysis (Fig. 1B) is consistent with orthology assignments made based on BLAST searches. Moreover, the resulting tree topology supports two distinct groupings within the ADMP-like molecules. The presence of two predicted ADMPs in a teleost suggests that the duplication giving rise to ADMP and ADMP2 preceded the split of the lineages leading to tetrapods and fish.

The temporal and spatial expression of ADMP2 mRNA

Expression of ADMP2 was first detectable by Northern blot analysis at late blastula stage (stage 9) and persisted through the

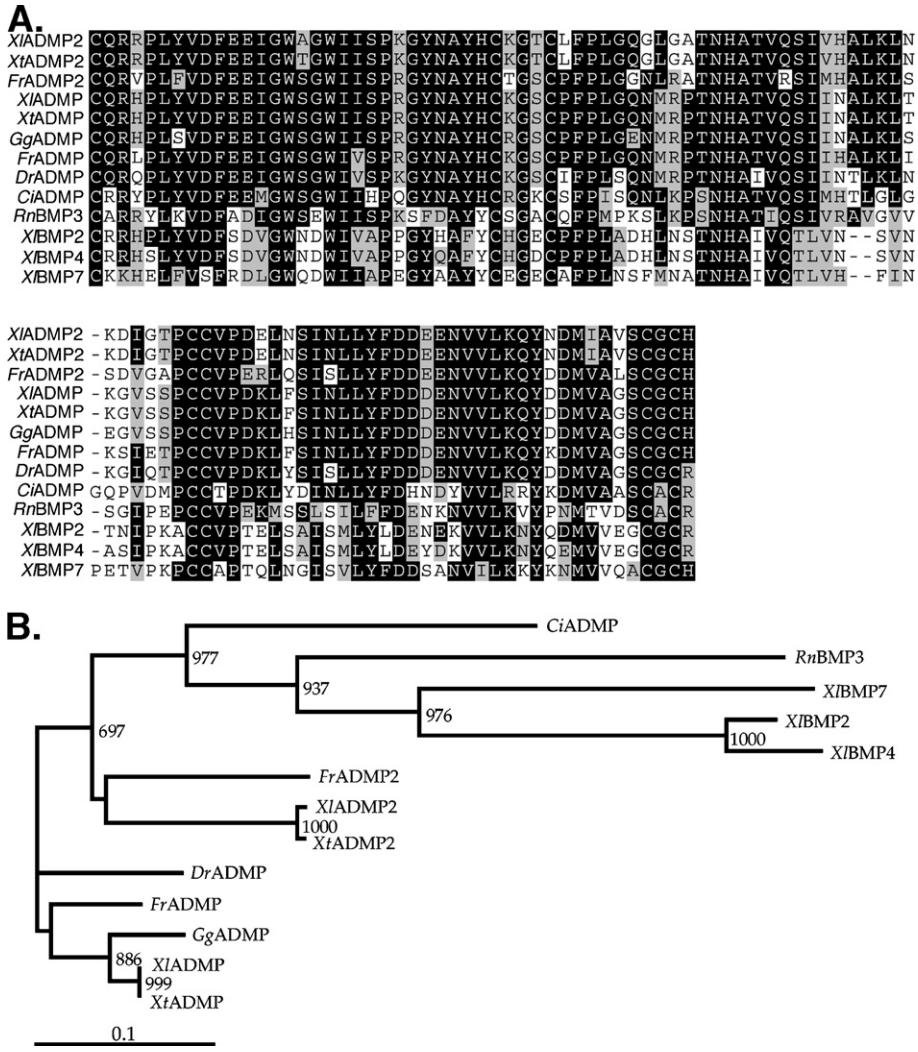

Fig. 1. The predicted ADMP2 protein. (A) Alignment of mature domain of *Xenopus laevis* ADMP2 (*Xl*ADMP2) with other members of the TGF-β family. (B) The schematic representation of the relationship between *Xl*ADMP2 and members of the ADMP and BMP families. The number at each branch point represents the relative incidence of that particular relationship during bootstrap resampling using 1000 replicates. The scale bar at the left bottom indicates an evolutionary distance of 0.1 amino acid substitution per position.

early neural fold stage (stage 15) (Fig. 2A). The spatial expression of ADMP2 was examined by in situ hybridization from early to late gastrula (stages 10 to 12; Nieuwkoop and Faber, 1994). At all stages, the hybridization signal for ADMP2 was very weak, and was best visualized in embryos that had been sectioned prior to hybridization. At stage 10 (Fig. 2B), a sagittal section shows strongest hybridization in a wedge of cells on the contra-organizer side (Kumano and Smith, 2002b) extending from surface of the marginal zone and narrowing towards the floor of the blastocoel. The surface marginal zone staining appears to correspond to a region that we have previously labeled as the "vegetal marginal zone," and which is found immediately vegetal to the band of Brachyury (Xbra) expression (Kumano and Smith, 2000). To investigate this further, stage 10.5 embryos were split along the organizer/contra-organizer axis into right an left halves. Figs. 2C and D show two halves of a single embryo, one half of which was hybridized for ADMP2 (Fig. 2C), and the other for Xbra (Fig. 2D). In comparing the two halves, it can be seen that the expression domains of Xbra and ADMP2 are very different, and apparently exclusive of each other. Significantly, the expression domain of ADMP2 extends below (i.e., vegetally) that of Xbra, and then extends upwards (i.e., animally) towards the blastocoel floor in cells that lie deeper than those expressing Xbra. The precise fate and germ layer origin of the ADMP2-expressing cells in the early gastrula are unclear. However, analysis of cell movements and fates in this region of the gastrula shows that mesoderm derives not only from the marginal zone, but also from the blastocoel roof (Ibrahim and Winklbauer, 2001). These authors describe ventral mesoderm as being derived from a similarly shaped wedge of tissue at stage 10.5, with the cells at the blastocoel floor comprising the leading edge (see: Fig. 10, Ibrahim and Winklbauer, 2001). However, the expression domain of ADMP2 is broader (i.e., deeper) than this, and includes endodermal cells. In the absence of the molecular probes required to precisely map the fates of ADMP2-expressing cells, the ADMP2 expression domain might best be referred to as mesendoderm.

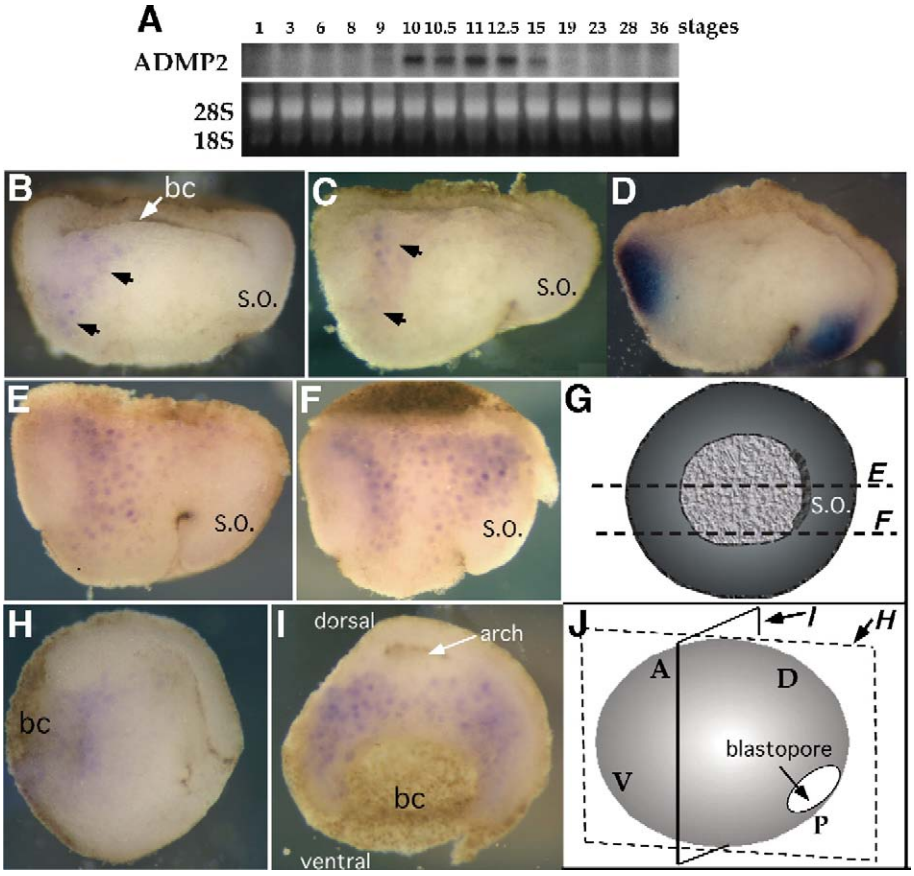

Fig. 2. Expression of ADMP2. (A) Expression of ADMP2 by Northern blotting of RNA from staged *Xenopus* embryos. Expression was first seen at stage 9 and persisted through the early neurula stage. Ethidium staining for 28S and 18S RNAs serves as a loading control. (B) In situ hybridization for ADMP2 in stage 10 embryo bisected sagittally. Arrows indicate general region of specific hybridization. (C and D) Two halves of bisected stage 10.5 embryo hybridized with either ADMP2 (C), or brachyury (D) (for the benefit of comparison, one image is inverted). (E and F) Sagittal (E) and para-sagittal (F) sections of stage 11 embryos hybridized for ADMP2. (G) Vegetal view of schematic stage 11 embryo indicating the approximate lines of bisection of embryos in panels E and F. (H and I) Stage 12 embryos hybridized for ADMP2. Dorsal is up and ventral is down for both embryos. Panel H shows sagittal section, while panel I shows transverse section. (J) Schematic view of stage 12 embryo showing the approximate plans of view in panels H and I. S.O.: approximate position of the Spemann organizer. bc: position of the blastocel.

At stage 10 and 10.5, ADMP2 expression is strongest on the contra-organizer side of the embryo, but positively staining cells can be seen extending towards, but not including, the Spemann organizer. Consistent with this, all expression of ADMP2 was abolished by injection of Xwnt8 RNA, which results in expansion of the Spemann organizer (Smith and Harland, 1991; Sokol et al., 1991) (not shown). The lateral extent of ADMP2 expression towards the Spemann organizer increases during gastrulation, and could be best visualized at stage 11 in sagittal (Fig. 2E) and para-sagittal (Fig. 2F) sections (see Fig. 2G for diagram of sections). In the sagittal section (i.e., running through the Spemann organizer), hybridization could only be detected on the contra-organizer side. However, in the para-sagittal section, hybridization on the Spemann organizer side of the embryo largely mirrored that seen on the contra-organizer side, with ADMP2 expressing cells found in deeper layers, and extending towards the blastocoel floor.

At late gastrulation (stage 12), the expression of ADMP2 in presumptive ventral mesendoderm is evident both in sagittal and transverse sections. During gastrulation, the leading edges of the involuting mass of cells converge at the ventral midline as they migrate along the walls and roof of the blastocoel (Keller, 1991). In the sagittal (Fig. 2H) section (see Fig. 2J for diagram of sections), ADMP2 can be detected in cells surrounding the collapsed blastocoel. Comparison of the ADMP2 staining pattern to fate maps of cells in this region, indicates that ADMP2 is expressed in both presumptive ventro-lateral mesoderm and endoderm. In an anterior transverse section that passes through the blastocoel (Fig. 2I), hybridization was detected in two “wings” of mesendoderm extending towards the ventral midline. No hybridization was detected in dorsal mesoderm, nor in endodermal cells making up the archenteron floor and roof.

ADMP2 regulation in animal caps and whole embryos

The basis for isolating ADMP2 was its induction in animal caps by Xnr2 in the absence of FGF signaling. To determine if FGF signaling plays a role in the regulation of ADMP2, animal caps were injected with Xnr2 RNA either with or without XFD RNA. We observed a much stronger induction of ADMP2 by the combination of Xnr2 and XFD compared to

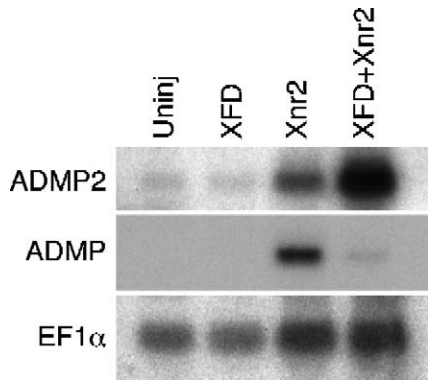

Fig. 3. Regulation of ADMP2 expression in animal caps and whole embryos. Northern blot of RNA from stage 10.5 animal caps injected with 1 ng of Xnr2 with or without 400 pg of XFD RNAs. The figure shows the results of hybridizations for ADMP2, ADMP and EF1α (loading control). Xnr2 RNA alone induces a low level of ADMP2 expression in animal caps, while co-injection of XFD RNA causes a much higher level of ADMP2 expression. ADMP expression, on the other hand, was inhibited by XFD.

Xnr2 alone (Fig. 3A). The negative regulation of ADMP2 by FGF contrasted with the expression of ADMP, which was induced strongly in animal caps by Xnr2 alone, but not at all by the combination of Xnr2 and XFD (Fig. 3). Thus, ADMP appears to be regulated similarly to Xbra and myoD, both of which are positively regulated by FGF (Hopwood et al., 1989; Smith et al., 1991; Cornell and Kimelman, 1994; LaBonne and Whitman, 1994; Kumano et al., 2001). In whole embryos, ADMP2 expression was also observed to be regulated by FGF signaling. Injection of XFD RNA resulted in increased ADMP2 expression, while injection of Xnr2 RNA alone had relatively little effect on ADMP2 expression. The induction of ADMP2 expression by Xnr2 in animal caps but not whole embryos may simply reflect de novo mesoderm induction in the former.

We have speculated that high levels of FGF signaling characterize presumptive dorsal mesoderm in the gastrula embryo, such as is found in the cells that express myoD and Xbra (Kumano and Smith, 2002b). Consistent with this, ADMP is expressed at stage 11 in late-involuted mesoderm cells in the Spemann organizer region (Moos et al., 1995), and at stage 13 in chordamesoderm (Dosch and Niehrs, 2000). The regulation of ADMP2, on the other hand, is more similar to that of genes Xnr2 and Xmenf, which are induced most strongly in animal caps by nodal and activin when FGF signaling is inhibited (Kumano and Smith, 2002a).

ADMP2 has a BMP-like activity

The phenotypes produced by injecting *Xenopus* embryos with high doses of RNA encoding either ADMP or ADMP2 are indistinguishable (Moos et al., 1995; Figs. 4D and E), and are characterized by an absence of head and dorsal structures such as somite, and a failure to elongate. This corresponds to a 0 in the DAI scale (Kao and Elinson, 1988) and is equivalent to the phenotype of BMP overexpression. Consistent with the DAI zero phenotypes, we observed that embryos which had been injected with BMP7, ADMP or ADMP2 had elevated globin expression but no muscle actin expression (Fig. 4A, lanes 2, 4 and 6 compared to lane 1). Although globin expression in the ADMP2-injected embryos was increased in amount, it was still restricted to the leading edge mesoderm (Fig. 4B, white arrow heads), similar to what is observed in UV-irradiated, BMP-injected and dnTcf3-injected embryos (Cooke and Smith, 1987; Kumano and Smith, 2000). The BMPs that give similar DAI zero phenotypes (BMP2, 4 and 7) all bind the type I BMP receptors (BMP2 and 4 bind to ALK3 and 6, and BMP7 binds to ALK2, 3 and 6; Graff et al., 1994; Suzuki et al., 1994; ten Dijke et al., 1994; Koenig et al., 1994). Based on the observation that a truncated ALK3 BMP receptor (tBR) was able to reverse the effects of BMP over expression, but not those of ADMP overexpression, it was proposed that ADMP does not act through the ALK3 BMP receptor (Dosch and Niehrs, 2000). In fact, recent results show that ADMP binds to the ALK2 receptor, but not ALK3 nor 6 (Reversade and De Robertis, 2005). Similarly to the observation by Dosch and Niehrs (2000), we observed that the co-injection of tBR did not dramatically change the phenotypes caused by ADMP2 overexpression (Figs. 4H and I compared to Figs. 4D and E, respectively). Moreover, co-injection of tBR restored muscle actin expression to the level of control embryos only in embryos that had been injected with BMP7 but not with ADMP or ADMP2 (Fig. 4A, lanes 3, 5 and 7 compared to lane 1). Some rescue of muscle actin was observed by co-injection of tBR in the ADMP2-injected embryos, and this may reflect some binding of ADMP2 to the BMP receptor which may result from overexpression. Globin expression, on the other hand, was eliminated by co-injection of tBR in all cases (Fig. 4A, lanes 3, 5 and 7 compared to lane 1), consistent with the fact that BMP signaling is essential for blood formation (Dale et al., 1992; Jones et al., 1992; Graff et al., 1994; Maeno et al., 1994a). In summary, ADMP2 does not appear to act mainly through the ALK3 receptor, and like ADMP may act through the ALK2 receptor.

Antisense morpholino "knockdown" of ADMP2

In order to study the role of ADMP2 during *Xenopus* embryogenesis, we generated an antisense morpholino oligonucleotide against ADMP2 (A-MO) for gene knockdown analysis (Heasman et al., 2000). In initial experiments, the efficiency of the A-MO in inhibiting translation of ADMP2 was tested using a green fluorescent protein (GFP)-tagged version of ADMP2 (A-GFP) in which the GFP coding region was added to a partial ADMP2 cDNA composed of the entire 5′ UTR and the first 6 nucleotides of the coding region. The A-MO spans nucleotides +5 to −20 with respect to the ATG of ADMP2. An in vitro synthesized A-GFP RNA was injected into both blastomeres of two-cell stage embryos that had previously been injected with either A-MO or a control morpholino oligonucleotide (c-MO). Proteins were extracted from the injected embryos at the neurula stage, and Western blot analysis with an anti-GFP antibody showed that translation of A-GFP was decreased in the embryos that had been injected with A-MO when compared to c-MO-injected embryos (Fig. 5).

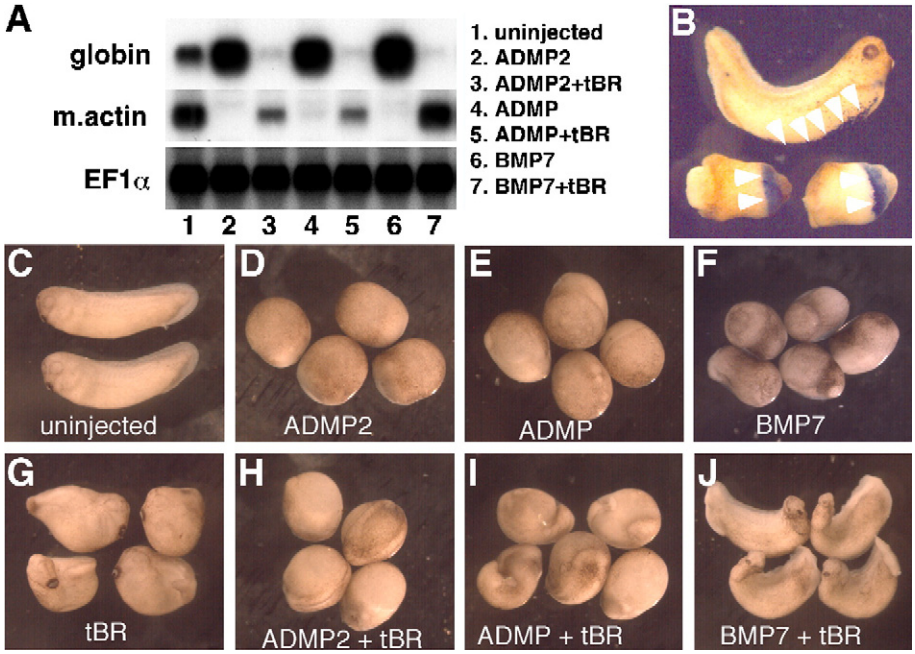

Fig. 4. Overexpression of ADMP2. (A) Northern blot of RNA from stage 32 sibling embryos of the ones shown in panels C–F and H–J. The figure shows the results of hybridizations for globin, muscle actin (m.actin) and EF1α (loading control). The expression of globin was increased by overexpression of ADMP2, ADMP or BMP7 RNA (lanes 2, 4 and 6 compared to lane 1), while that of m.actin was virtually eliminated by the same treatment (lane 2, 4 and 6 compared to lane 1). The level of m.actin expression in the BMP7-injected embryos was rescued to normal levels by co-injection of tBR RNA (lane 7 compared to lane 1). However, in the ADMP2 or ADMP-injected embryos, co-injection of tBR RNA failed to rescue m.actin expression (lanes 3 and 5 compared to lane 1). (B) The phenotype and globin expression by in situ hybridization in ADMP2-overexpressed embryos. The expression of a blood marker, globin, is normally detectable in the ventral-most region of the tailbud stage embryo at stage 32 (white arrowheads in top embryo). Overexpression of ADMP2 caused a typical DAI 0 phenotype (two bottom embryos). The expression of globin was restricted in the leading edge mesoderm of these embryos (white arrowheads) at the equivalent of stage 32. (C–J) Rescue experiments by co-injection of tBR RNA. Overexpression of either ADMP2, ADMP or BMP7 RNA caused a similar DAI 0 phenotype (C–E compared to B). However, only the phenotype caused by overexpression of BMP7 was rescued by co-injection of tBR RNA (G–I), suggesting that ADMP2 and ADMP do not bind the BMP receptor. (G) The phenotype of tBR RNA-injected embryos. All the embryos shown are at stage 32.

ADMP2 is essential for ventral mesoderm formation

We next examined the effect of A-MO on *Xenopus* embryonic development. Morpholino oligonucleotide injections were performed at the 4-cell stage into two blastomeres on either the Spemann organizer side or the contra-organizer side. The A-MO and c-MO-injected embryos were analyzed by in situ hybridization for genes expressed in dorsal mesoderm (myoD), lateral mesoderm (Xnr1 and Xlim1), ventral mesoderm [globin and SCL (blood); Nkx2.5 and cardiac troponin I (TnIC) (heart)] and endoderm (endodermin) (Table 1). The A-MO-injected embryos at both doses tested (5 and 10 ng/blastomere) appeared largely normal, and had no gross malformations, such as axial defects. Furthermore, the expression of myoD, Xlim1, Xnr1 and endodermin in embryos injected with 10 ng/blastomere of A-MO was indistinguishable from control embryos (Figs. 5B through G, Figs. 7L and M). Because the A-MO does not create a null – rather it "knocks down" the gene product – we cannot conclude that ADMP2 plays no role in the development of these tissues.

However, further analysis of these embryos by in situ hybridization revealed that 97% of the embryos ($n=30$) injected anteriorly with 10 ng/blastomere A-MO had greatly reduced expression of the heart marker, Nkx2.5, and 93% of the embryos ($n=29$) injected posteriorly had a strong reduction in expression of the blood marker globin in the posterior ventral blood islands (VBIs) (Table 1). The range of reduction observed in the expression of these marker genes ranged from moderately reduced to faint residual staining (see Figs. 6 and 7). These effects on Nkx2.5 and globin expression by A-MO were dose-dependent, and injection of 5 ng/blastomere of A-MO decreased the percentage of affected embryos in both cases to 64% and 44%, respectively (Table 1).

ADMP2 and heart development

The *Xenopus* heart undergoes extensive morphogenesis during the tailbud stage (Mohun et al., 2000). We have investigated the effect of ADMP2 knockdown on heart formation at stage 32 because at this stage the myocardium has formed a tube in all but the most anterior region (Mohun et al., 2000). Nkx2.5 was used as a specific marker for heart at this stage (Figs. 6A through F) (Tonissen et al., 1994). In transverse sections of control embryos through the posterior heart, Nkx2.5 expression was observed in the tube of the myocardium (Fig. 6D, white arrowhead), and the pericardial roof (Fig. 6D, black arrowheads). The pericardial cavities lateral to the tube can be also recognized (Fig. 6D, arrow). In A-MO-injected embryos, however, the staining for Nkx2.5 was faint, and it appeared as though the heart tube had not formed (Figs. 6B and E). The effect of A-MO on heart formation was specifically due to

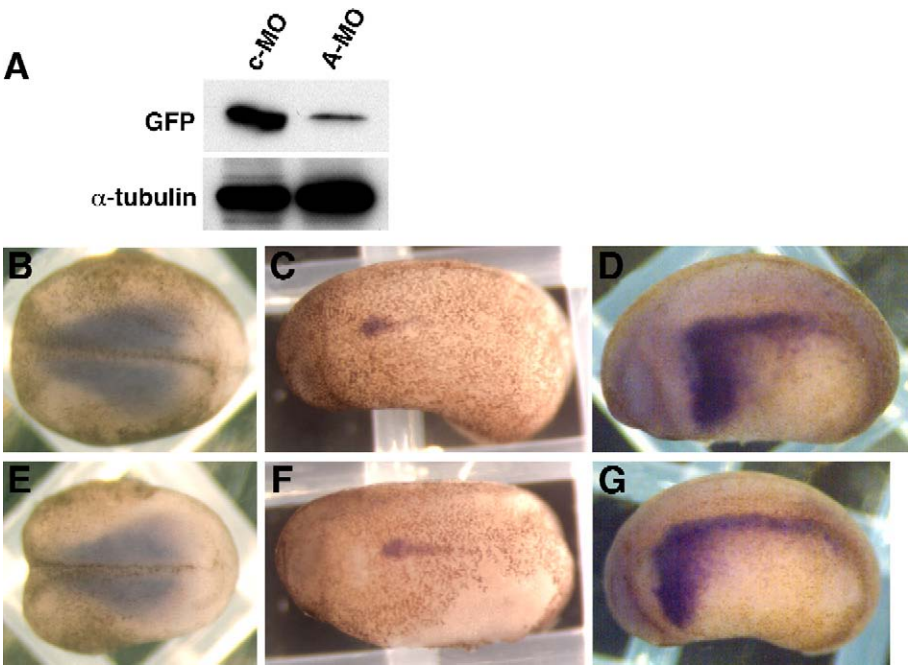

Fig. 5. (A) Inhibition of translation by an ADMP2 antisense morpholino oligonucleotide. Western blot of proteins extracted from stage 14 embryos that have been injected separately with 100 pg of RNA encoding an ADMP2/GFP fusion protein and either 10 ng of a control (c-MO) or antisense ADMP2 morpholino oligonucleotide (A-MO). Shown is the result of immunoblotting using an anti-GFP antibody and an anti-alpha-tubulin antibody (loading control). (B–G) Loss-of-function analysis of ADMP2. Control embryos (B, C, D), and embryos (E, F, G) injected with an antisense ADMP2 morpholino oligonucleotide (A-MO) probed for expression of a somite marker, *XmyoD* (B, E), a pronephros marker, *Xlim1* (C, F) or a lateral plate marker, *Xnr1* (D, G). (B, E) Stage 14 embryos. Anterior is to the left. Dorsal views. (C, D, F, G) Stage 24 embryos. Lateral views. Anterior to the left.

inhibition of ADMP2 translation, as co-injection of a modified version of ADMP2 RNA (ADMP2 Δ-5′ UTR) devoid of its 5′ UTR rescued the phenotype caused by injection of A-MO (Figs. 6C and F; Table 2). The amount of ADMP2 Δ-5′ UTR RNA injected in this rescue experiment (5 pg×2) did not cause any phenotypes when injected alone (data not shown). The expression of a myocardium specific marker, TnIc (Drysdale et al., 1994), was also reduced at this stage (Fig. 6H compared to G; Table 1). The hearts in A-MO-injected embryos at stage 46 were much smaller than those of control embryos, although they could beat (data not shown). These results suggest that ADMP2 plays a role in specifying the heart.

Table 1
Antisense ADMP2 morpholino oligonucleotide

Markers	Uninjected	c-MO (5 ng)	c-MO (10 ng)	A-MO (5 ng)	A-MO (10 ng)
Globin (P)	–	90% (n=21)	85% (n=20)	56% (n=102)	6.9% (n=29)
Globin (A)	–	100% (n=23)	100% (n=23)	84% (n=51)	43% (n=14)
SCL (P)	–	–	85% (n=26)	–	28% (n=47)
Nkx2.5 (A)	–	100% (n=23)	86% (n=22)	36% (n=93)	3.3% (n=30)
TnIC (A)	–	94% (n=16)	–	18% (n=33)	
Xlim1 (P)	100% (n=13)	–	–	–	92% (n=13)
Xnr1 (P)	86% (n=50)	–	–	–	80% (n=44)
MyoD (P)	100% (n=13)	–	–	–	100% (n=10)
Endodermin (P)	100% (n=36)	–	–	–	100% (n=30)

Results are reported as the percentage of embryos showing an expression of the particular gene that is indistinguishable from the previously published pattern. A-MO: antisense ADMP2 morpholino oligonucleotide; c-MO: control morpholino oligonucleotide. P: posterior injection, A: anterior injection.

ADMP2 and ventral blood island development

The VBIs form along the anterior/posterior axis of the belly of the *Xenopus* larvae. Injection of the A-MO either anteriorly or posteriorly resulted in a corresponding localized reduction in the level of αT3 globin expression at stage 32 (Figs. 7A through F and Figs. 7I through K). While 5 ng/blastomere of A-MO was sufficient to disrupt globin posteriorly, disruption of anterior globin expression was only observed at 10 ng/blastomere (Table 1; Figs. 7A, B, and D through F). The reason for this difference is not clear, particularly since 5 ng/blastomere A-MO injected anteriorly disrupted expression of heart markers (Table 1). The effect of A-MO on VBI development was specifically due to the knockdown of ADMP2 as co-injection of ADMP2 Δ-5′ UTR RNA with A-MO rescued globin expression (Figs. 7C and K, arrowheads; Table 2). The amount of ADMP2 Δ-5′ UTR RNA injected in this rescue experiment (10 pg×2) did not alter morphology when injected alone (data not shown).

αT3 globin is a terminal differentiation marker for the primitive erythroid lineage, and the disruption of its expression by A-MO might indicate a requirement for ADMP2 for certain blood cell lineages only. However, we observed that expression

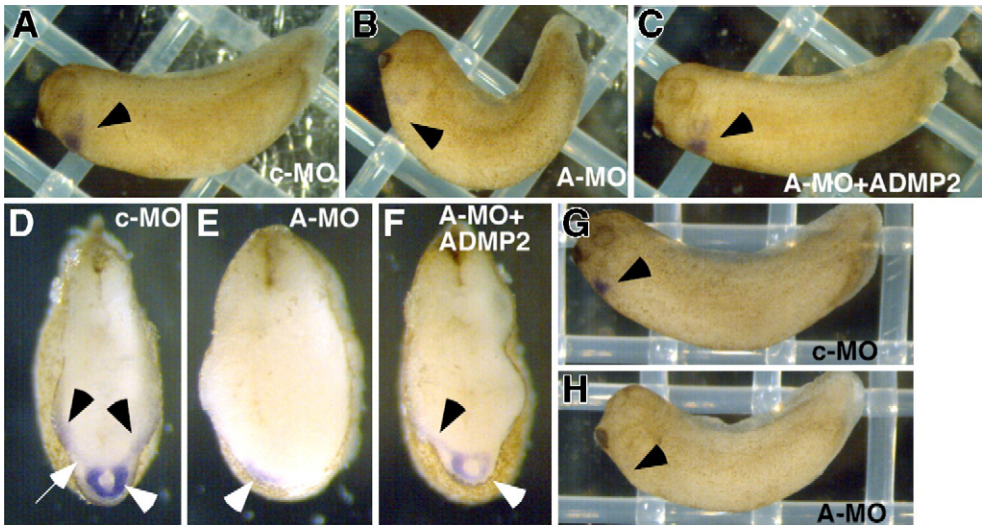

Fig. 6. Loss of ADMP2 and heart formation. (A and B) Injection of 10 ng of an antisense ADMP2 morpholino oligonucleotide into each of the two anterior blastomeres of a 4-cell stage embryo inhibited the expression of the heart marker Nkx2.5 (c-MO: control morpholino; A-MO: antisense ADMP2 morpholino). (C) This effect was reversed by co-injection of 5 pg of ADMP2 Δ-5′ UTR RNA (total 10 pg) (black arrowheads). (D) Transverse sections of c-MO-injected embryos showed that Nkx2.5 was expressed in a tube of myocardium (white arrowheads) as well as the pericardial roof (black arrowheads in panel D). (E) A-MO-injected embryos, on the other hand, showed only faint expression of Nkx2.5 (white arrowhead in panel E), and the heart tube was not formed. (F) Rescue of the A-MO phenotype, as in panel C. (G, H) Anterior injection of 10 ng A-MO also reduced the expression of TnIC, another heart marker (black arrowhead in panel K compared to that in panel J) (82%; Table 1).

of the gene SCL, which is expressed throughout the early hematopoietic stem cells (Mead et al., 1998; Davidson and Zon, 2000), was reduced by A-MO at stage 24 (Fig. 7H compared to G). Thus the entire primitive hematopoietic lineage appears to be disrupted by injection of A-MO.

While the inhibition of globin expression by A-MO was consistently observed, faint expression of globin persisted even at the higher level of injected A-MO in many embryos (for example, Fig. 7B). In a transverse section of an A-MO-injected embryo at the level of the posterior VBIs, globin was observed in a thin layer just underneath the ventral ectoderm (Figs. 7J and L), while globin expression in c-MO-injected embryos extended deeper into the mesodermal layer (Figs. 7I and L). The most likely explanations for the persistence of low level globin expression in A-MO-injected embryos are that the A-MO may not be completely effective in inhibiting ADMP2 translation (see Fig. 5), or that some blood is induced by an ADMP2-independent pathway.

ADMP2 and BMP signaling in blood induciton

BMP signaling and its modulation are essential for many processes in early vertebrate development, and alterations of BMP signaling, either by overexpression or loss of function, result in profoundly abnormal development (Dale et al., 1992; Jones et al., 1992; Graff et al., 1994; Suzuki et al., 1994). For the development of the VBIs, ectodermally derived BMP signaling is proposed to induce primitive blood in the underlying mesoderm (Maeno et al., 1994b; Kumano et al., 1999; Kikkawa et al., 2001; Walmsley et al., 2002; Walters et al., 2002). The fact that the A-MO-injected embryos appeared normal, with the exception of ventral mesoderm derivatives, indicates that there is no widespread disruption of BMP signaling. Nevertheless, we investigated whether exogenous BMP7 would be able to rescue VBI development in ADMP2 depleted embryo. Because BMP overexpression causes a widespread disruption of development, we first tested a low dose of BMP7 that does not give a strong phenotype. At this dose of BMP7 (20 pg RNA), no elevation or rescue of globin expression was observed (data not shown). We next tested a second and higher dose of injected BMP7 RNA (1 ng RNA). Injection of 1 ng of BMP7 RNA alone into embryos resulted in embryos with a typical DAI 0 phenotype and high levels of globin expression in the descendents of the leading edge mesoderm (Fig. 7N). However, embryos coinjected with the A-MO and 1 ng BMP7 RNA had greatly reduced levels of globin expression (10/16) relative to the embryos injected with BMP7 alone, although the embryos still had DAI 0 phenotypes (Fig. 7M).

Discussion

ADMP2 was isolated from a library that was enriched for genes induced in *Xenopus* animal caps by the mesoderm inducer Xnr2 in the presence of XFD (Kumano and Smith, 2002a). The rationale for this screen is that in animal caps injected with Xnr2 alone, dorsal mesoderm is predominantly induced, while coinjecton of XFD to inhibit endogenous FGF signaling results in the strong induction of ventral mesoderm in the absence of dorsal mesoderm (Kumano et al., 2001). We reasoned that genes induced in animal caps by the combination of XFD and Xnr2 would likely be expressed in the presumptive ventral mesoderm, and analysis of their function may provide new clues to understanding the pathway of ventral mesoderm induction.

Table 2
Rescue of A-MO-injected embryos

Markers	Uninjected	A-MO-injected	A-MO/ADMP2-Δ-5′UTR co-injected
Globin (P)	96% (n=26)	9.1% (n=33)	77% (n=31)
Nkx2.5 (A)	100% (n=29)	26% (n=39)	73% (n=37)

Embryos were injected with 10 ng/blastomere of A-MO either with or without coinjected ADMP2 Δ-5′UTR RNA (5 pg RNA for anterior injection and 10 pg for posterior injection). Results are reported as the percentage of embryos showing an expression of the particular gene that is indistinguishable from the previously published pattern. A-MO: antisense ADMP2 morpholino oligonucleotide. P: posterior injection, A: anterior injection.

ADMP2 and the origin of ventral mesoderm

ADMP2 is most closely related to ADMP by sequence and overexpression phenotype. However, the two genes are clearly distinguished by their expression patterns. ADMP is expressed in the Spemann organizer during gastrulation, and subsequently in the axial mesoderm of neurula stage embryos (Moos et al., 1995; Dosch and Niehrs, 2000). In contrast, ADMP2 is expressed during early- and mid-gastrulation in a swath of cells which extend from surface of the marginal zone towards the blastocoel floor. The assignment of precise fates to the ADMP2-expressing cells in the gastrulating embryo is not simple. The ADMP2 expression domain is distinct from the Xbra-expression domain, with ADMP2-expressing cells found both vegetally and deeper than the Xbra-expressing cells. We have previously shown that cells which express Xbra during the gastrula stage do not populate the belly of the tadpole, but rather end up exclusively in the dorsal mesoderm (Kumano et al., 2001). Studies that have examined mesoderm migration and fate in deep layers of the gastrulating embryo have described leading edge mesoderm at mid-gastrulation as extending up in a wedge from the surface to the blastocoel floor, a pattern similar to that reported here for ADMP2 expression (see: Fig. 5F, Keller, 1991; and Fig. 10 of Ibrahim and Winklbauer, 2001). However, the reports differ on the pre-gastrula origin of the cells

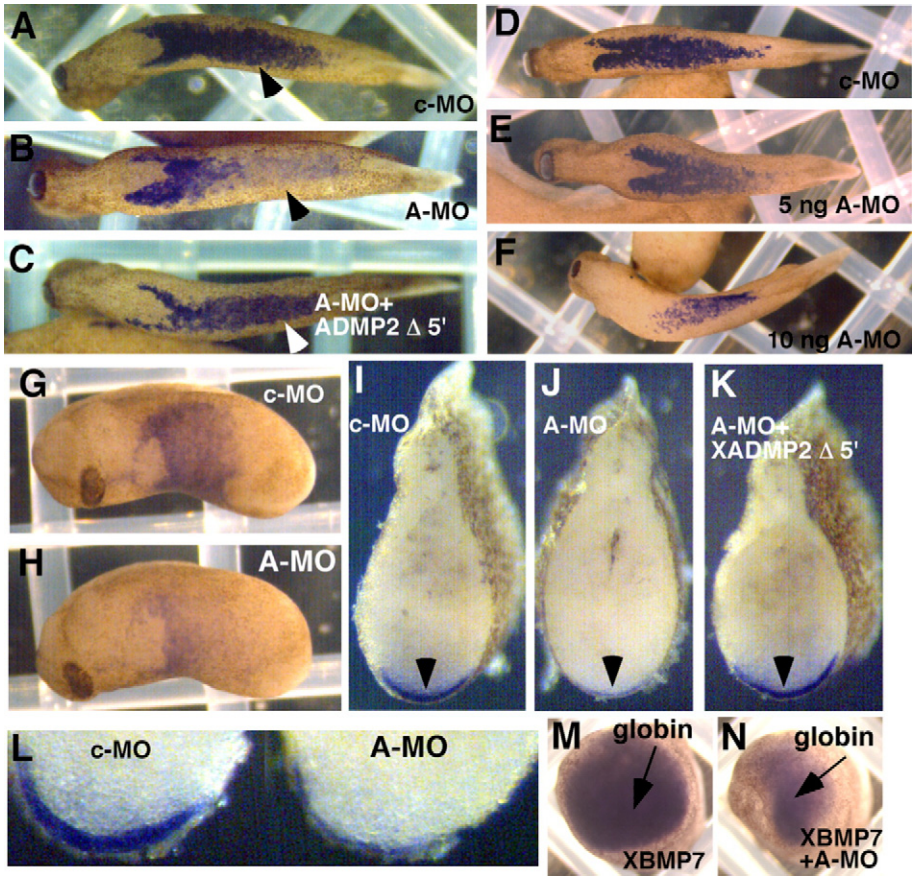

Fig. 7. ADMP2 loss and blood formation. (A and B) Posterior injection of 10 ng of antisense ADMP2 morpholino oligonucleotide (A-MO) into each of the two posterior blastomeres at the four cell stage inhibited the expression of globin in the posterior ventral blood islands (c-MO: control morpholino). (C) Co-injection of 10 pg of ADMP2 Δ-5′ UTR RNA (total 20 pg) with A-MO restored globin expression (white arrowhead). (D–F) Anterior injection of 5 and 10 ng/blastomere of A-MO. While the 5 ng dose inhibited globin expression when injected posteriorly (E), no reduction in globin was observed at this dose anteriorly. At 10 ng (F) a strong reduction in globin expression anteriorly was observed. (G and H) The expression of SCL, an early blood marker, was also reduced by injection of 10 ng of A-MO posteriorly when examined at stage 24. (I and J) Transverse sections at the level of the posterior blood islands show that the expression of globin was decreased to a thin layer in the A-MO-injected embryos (black arrowhead in panel J compared to that in panel I). (K) In embryos co-injected with A-MO and ADMP2 Δ-5′ UTR RNA, the globin-expression extended deep into the mesodermal layer. (L) Enlarged view of ventral mid-line showing globin expression in either c-MO (left) or A-MO-injected embryos. (M, N) Coinjection of 10 ng A-MO into each blastomere at four cell stage reduced globin expression in response to BMP7 RNA injection (1 ng per embryo). Note that both control (M) and A-MO-injected embryos (N) have the same DAI 0 phenotype.

at the extreme leading edge, with Keller depicting their origin at the vegetal marginal zone, and Ibrahim et al. placing their origin at the blastocoel floor. In either case, the mid-gastrula expression domain of ADMP2 appears to include presumptive ventral, but not dorsal mesoderm. However, the ADMP2 expression domain in the gastrulating embryo extends very deep, and includes endodermal precursors. Further analysis of the fates of ADMP2-expressing cells, particularly with a promoter reporter construct as was done for Xbra (Kumano et al., 2001), may help shed new light on the origin and induction of ventral mesoderm.

ADMP2 is essential for ventral mesoderm development

Heart formation

Injection of morpholino oligonucleotides against ADMP2 disrupted heart development and inhibited the expression of the heart markers Nkx2.5 and TnIc at stage 32. The development of the heart in *Xenopus* is a complex process involving inductions from neighboring tissues. It appears that wnt antagonists from the Spemann organizer followed by signals from cerberus-expressing endoderm cells (Schneider and Mercola, 1999) during gastrulation initiate Nkx2.5 expression (Schneider and Mercola, 2001). Tissue isolation experiments demonstrate that signaling from the Spemann organizer is largely complete by stage 10, while the endodermal signals remain active even after stage 10.5 (Nascone and Mercola, 1995). The expression of Nkx2.5 is then maintained, but not initiated, by BMP signaling (Shi et al., 2000; Walters et al., 2001). Since ADMP2 is expressed neither in the Spemann organizer region nor in cerberus-expressing endoderm cells during gastrulation, it is unlikely that this gene acts as an initiator of Nkx2.5 expression. Instead, it is more likely that ADMP2 is necessary for specification of the heart at a later stage within the leading edge mesoderm. As both the heart and the VBIs are from this mesoderm and both are affected by morpholino injection, ADMP2 may play a role in specifying this entire region of mesoderm, from which the heart is subsequently derived.

VBI formation

Injection of antisense morpholinos to ADMP2 inhibited the expression of VBI markers SCL at stage 24, and globin at stage 32. This effect is not due to a loss of presumptive ventral mesoderm, nor to a failure of the cells to migrate to their proper location. Rather, ADMP2 more likely contributes directly to blood induction. The inductive factors most strongly associated with VBI induction are the BMPs (Dale et al., 1992; Jones et al., 1992; Graff et al., 1994; Maeno et al., 1994a,b, 1996; Zhang and Evans, 1996; Huber et al., 1998; Kumano et al., 1999; Walmsley et al., 2002). Early models of mesoderm patterning proposed that gastrula stage BMP signaling was sufficient for VBI induction, and that genes downstream of gastrula BMP signaling, such as Xvent 1, were in the pathway of VBI induction (Huber and Zon, 1998; Davidson and Zon, 2000). Furthermore, the blood inducing activity of BMP has been taken as evidence in favor of the Spemann organizer model of mesoderm patterning because the region of the gastrula marginal zone with the highest level of BMP expression, the pole of the marginal zone opposite the Spemann organizer, was thought to correspond exactly to location of presumptive primitive blood cells. However, further analysis has shown that blood cells originate from a much broader region of the marginal zone, including domains with very low BMP expression at gastrulation (Tracey et al., 1998; Lane and Smith, 1999; Mills et al., 1999; Ciau-Uitz et al., 2000). This apparent paradox in the BMP requirement for blood induction appears to be explained by observations that the induction of primitive blood may not occur until the end of gastrulation when ventral mesoderm meets up with BMP-producing ectoderm. It is proposed to be this ectodermally derived BMP that is essential for primitive blood induction, not the gastrula stage BMP within the marginal zone (Maeno et al., 1994a,b; Kumano et al., 1999; Kikkawa et al., 2001; Walmsley et al., 2002; Walters et al., 2002).

A two-step model for the induction of the VBIs has been proposed (Kumano et al., 1999; Kikkawa et al., 2001; Kumano and Smith, 2002b; Walmsley et al., 2002). In the first step, presumptive ventral mesoderm is induced by general mesodermal inducers. In the second step, BMP signaling from ectoderm at late gastrulation induces the ventral-most mesoderm to differentiate as VBI. Previously we have shown that the leading edge mesoderm is uniquely able to respond to BMP signaling to differentiate as VBI. For example, when BMP is overexpressed, the total amount of globin increases, yet globin expression remains restricted to the leading edge mesoderm (Fig. 7O; Kumano and Smith, 2000). FGF appears to play a role in defining domains within the mesoderm that have differential competence to respond to primitive blood inducers. For example, inhibition of FGF signaling leads to widespread globin expression in the mesoderm-forming marginal zone (Kumano and Smith, 2000). Nevertheless, the expansion of the blood forming territory by FGF inhibition remains BMP dependent, consistent with the two-step model. We have shown here that loss of globin expression in embryos injected with an ADMP2 antisense morpholino oligonucleotide cannot be rescued by BMP, suggesting that ADMP2 is involved with the first step in VBI induction, and that, in the absence of ADMP2, presumptive ventral mesoderm is not competent to be induced to primitive blood.

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